XANTHINE OXIDASE, ACID PHOSPHATASE, RIBONUCLEASE, AND PROTEASE

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There is a variety of enzymes in milk. In most cases there are components in milk that can be acted on by these milk enzymes. The components on which particular enzymes act are called substrates. Enzymes are catalysts that can bring about chemical changes, changes that as a rule are quite specific for each enzyme. With both enzymes and their substrates present in milk, it is surprising that there are few readily observed changes in milk directly attributed to enzymes other than the action of lipase (13). This lack of changes due to enzymes in part might be due to the usual use of pasteurization in the marketing of milk. Many enzymes are heat-sensitive and lose their catalytic activity when heated. Also, the reason there is not more in dairy chemistry literature about the milk enzymes in relation to milk stability must mean that these enzymatic changes are not very obvious and do not alter the physical stability or flavor of beverage milk. The action of milk enzymes on the components of milk, however, deserves more attention. Heat-inactivated enzymes can regain their activity under some conditions. Enzymes are catalysts, and hence are not used up in the reactions they bring about; therefore, their action can continue for a long time. The effect of enzymes accordingly might be expected to show up in stored milk products. Here a difficult problem will be to distinguish between changes due to the enzymes inherent in milk and changes due to enzymes produced by microorganisms present in milk. These considerations have been kept in mind in preparing the following survey on the action of several milk enzymes on components of milk.

The first milk enzyme to be considered is xanthine oxidase. Although enzymes are relatively specific in the compounds they act on, xanthine oxidase appears to be an exception. Xanthine oxidase will act on such different compounds as the purines, of which xanthine is an example, and aldehydes. Aldehydes are oxidized to acids with the hydrated aldehyde as an intermediate step (21). A similar hydration occurs at a carbon-nitrogen double bond in xanthine and, on subsequent transfer of the hydrogens, uric acid is formed. Thus, the action of xanthine oxidase on purines and aldehydes is more similar than one would anticipate from a quick look at the formulas. The hydrogens, when activated by the enzyme, can be transferred to other molecules such as redox dyes as well as to oxygen. The decolorization of methylene blue by milk with one of the substrates by activation of the hydrogens and transfer to the methylene blue provides a satisfactory assay of this enzyme. Actually, this enzyme was first discovered in milk when formaldehyde and methylene blue were added to fresh milk and the latter was seen to be decolorized. For a long time this was known as the Schardinger enzyme, after its discoverer, but it was subsequently shown to have a much broader range of activity and was given the name xanthine oxidase. Actually, the amount of substrates for this enzyme in milk appears to be quite limited, for the reduction of methylene blue or other similar dyes by fresh, high-quality milk is very slow. The addition of a substrate such as xanthine, however, will produce a very rapid decolorization (7). This enzyme contains a flavin as a co-enzyme, and molybdenum. The variation in the xanthine oxidase content of milk produced in different localities apparently is related to the molybdenum content of the forage consumed by the cow (14). Of most importance for the present discussion is the heat stability of this enzyme. Does it persist in milk after heat treatment? This is one of the enzymes in milk that is activated by variation in temperature [storage at 38 F (4 C); heating 5 min at 158 F (70 C)] and by other physical treatments such as homogenization, and treatment with commercial proteolytic and lipolytic enzymes. Gudnason and Shipe (10) have thoroughly investigated this phenomenon, particularly the increase in activity of xanthine oxidase as the result of storage at 38 F (4 C). Corresponding decreases of the oxidase in the fat phase and increases in the skim phase occurred. At higher temperatures, xanthine oxidase is more stable than alkaline phosphatase (24) and less stable than lactoperoxidase (12). Thus, under conditions [140 F (60 C) for 15 min] that completely inactivated the alkaline phosphatase, only 40% of the xanthine oxidase was inactivated (24). These stability tests were performed with milk. As a rule, the purified milk enzymes are considerably more heat-labile. That is, the presence of other proteins, in a considerably higher concentration than the enzymes, has a protective effect.

The storage history of milk is important for the heat stability of xanthine oxidase. Gudnason and Shipe (10) have found that the heat sensitivity of xanthine oxidase is increased on storage and on homogenization, and thought

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this due to a disintegration of the microsomes associated with the fat globule membrane.

This enzyme, xanthine oxidase, because of its broad range of activity, has been considered as perhaps responsible for some of the oxidative deterioration in the flavor of milk. The evidence, however, has been conflicting. Recent work from several universities (19, 20) could not establish a correlation between xanthine oxidase content and oxidative flavor, based on chemical test or taste panel scores. It was recommended (20) that milk for such comparison be held for at least 12 hr, so that the xanthine oxidase reaches its maximum, constant value.

A recent paper by Greenbank and Pallansch (9) reported the reactivation of the xanthine oxidase in storage of dried whole milk products. This occurred with milks heated in a Mallory type heater at 195 F for 15 sec, that is, a high-temperature, short-time treatment. Additional studies are desirable to determine the range of treatments under which reactivation will occur. Will it occur with fluid milk, for example? The xanthine oxidase is concentrated in the cream portion of milk and is considerably more resistant to the effect of heat in cream. Thus, after pasteurization of cream, only 2.3% of the xanthine oxidase had been inactivated, whereas with whole milk it was 23.6% and in skimmilk 27.9%. Xanthine oxidase, however, in spite of the relative instability in skimmilk, was still present in pilot plant and commercial spray-dried low-heat (150–170 F) skimmilk powders, whereas none was present in high-heat (180–215 F) powders. The authors suggest that xanthine oxidase assays might provide a rapid method for estimating the heat treatment that nonfat powders had received. From studies done to date it appears that xanthine oxidase has the potential of causing oxidized flavors, but additional studies are desirable with this relatively heat-stable enzyme.

The next milk enzyme to be considered is acid phosphatase. The enzyme hydrolyzes phosphate esters just as does alkaline phosphatase, but its distinguishing feature is indicated by its name. The enzyme has its maximal activity in more acid solutions; namely, at about pH 5. There are low molecular weight phosphate esters in milk and the phosphatases can split them. Graham and Kay (8) did a phosphorus partition on milk in storage and found that the free inorganic phosphate increased at the expense of the acid-soluble (that is, low molecular weight) ester phosphate, whereas the other phosphate fractions (acid-insoluble, principally casein and lipide) remained fairly constant. This small change in the amount of low molecular weight phosphate esters probably has no influence on the flavor or stability of milk. Recent studies have shown, however, that both the alkaline phosphatase (23) and the acid phosphatase (4) of milk can split the phosphate from casein. The phosphate in casein, that is, the chemically bound part, is bound to the amino acid, serine. Serine is an hydroxy amino acid and the phosphate is bound to the hydroxyl group. The serine is bound to the other amino acids of casein through its carboxyl and amine groups. The enzyme splits the ester bond with an uptake of one molecule of water to give the separated components. The acid phosphatase is more likely to hydrolyze the casein in milk than the alkaline phosphatase. First, it is a very heat-stable enzyme. Mullen (18), who first studied this enzyme in milk, observed that 80-90% of the acid phosphatase activity of whole milk remained after heating at pasteurization conditions of 145 F (62.8 C) for 30 min. This stability has been confirmed by others (2). In addition, although there is considerably more alkaline phosphatase than acid phosphatase in milk, at the pH of milk the latter has more activity with casein (4). This possible loss of phosphate from casein is of great interest to the researcher who would like to characterize the caseins by their phosphorus content. Certainly, it would be of interest to compare the phosphorus content of casein from freshly drawn milk with that stored for some time, to see whether there might be a decrease in the phosphorus content. The loss of phosphate from casein would be of interest from the standpoint of physical stability. Casein precipitates at pH 4.7, a pH at which it is a neutral molecule. With loss of the acidic phosphate molecule it would precipitate at a higher pH value. Although the change in this pH value might be small, small changes sometimes are very important in a system as complex as milk. This appears to be an area for further exploration.

The next milk enzyme to be considered is ribonuclease. This enzyme hydrolyzes ribonucleic acid to the component nucleotides. Ribonucleic acid is a phosphate-ribose chain with the organic bases, the purines and pyrimidines, as side groups. The phosphate diesters are split by hydrolysis, but no free inorganic phosphate is formed. Hydrolysis of the phosphate ester occurs only where the side-group base is a pyrimidine. Subsequently, successive hydrolysis occurs to give the base-ribose component with one molecule of phosphate in the 3 position of the ribose. This compound is called a nucleotide. Ribonuclease is present in cow's milk in relatively large amounts (3), but surprisingly it is not present, or present only in traces, in goat's milk, human milk, sheep milk, and guinea pig milk (5). The ribonuclease in cow's milk has been obtained in pure form and amino acid composition and immunological methods indicate it is identical with the ribonuclease obtained from bovine pancreas (5). Biochemists have devoted a great deal of study to this enzyme because of its relatively low molecular weight (13,700) and basic nature (isoelectric point of pH 7.8). Its structure, the sequence of the amino acids in the peptide

chain, is now known. But what about ribonuclease in relation to milk? It is a very heat-stable enzyme. Little of the milk ribonuclease is lost by precipitating the casein with acid, so whey can be used for the heat stability studies. Whey adjusted to pH 3.5 lost none of its ribonuclease activity when heated at 194 F (90 C) for 20 min; at pH 7 all of the activity was lost (50% was still present after heating 5 min) (3). This marked influence of pH on the heat stability had been observed for the pancreatic ribonuclease also. In any case, one can conclude that ribonuclease will still be present in milk after pasteurization. Are there nucleic acid components in milk on which ribonuclease can act? Morton has presented evidence (16, 17) that the lipoprotein complex of the fat globule membrane contained microsomes. Microsomes are particulate cellular components, rich in enzymes (xanthine oxidase, alkaline phosphatase, etc.) and they also contain ribonucleic acid. Ribonucleic acid could not be found (25) in fat globule membrane prepared from commercial milk, but Bailie and Morton state that it can readily be found in fresh milk and particularly in milk from individual cows (1). These authors feel that the enzymes in milk, and this would be principally ribonuclease, account for the disappearance of the nucleic acid later. This appears to be an area for further exploration. Certainly, the fat globule membrane has an important role in the stability of the cream emulsion and one might expect that the presence or absence of the highly charged ribonucleic acid would have a considerable influence on the properties of the emulsion.

The next and last milk enzyme to be discussed is protease. This enzyme catalyzes the hydrolysis of the peptide bond with the appearance of an amino and a carboxyl group. Proteases split proteins into smaller fragments and even release free amino acids. Proteases have considerable specificity; trypsin, for example, hydrolyzes peptide bonds adjacent to the basic amino acids arginine and lysine (carboxyl end). The specificity of the milk protease has not been established. The main difficulty in studying the protease in cow's milk is that it is there at a low level. In fact, it has taken careful work to establish that the proteolysis observed in milk is not due to bacteria (11). Very much protease in milk would be incompatible with one of the functions of milk for the calf. The first milk, the colostrum, is the carrier of antibodies for the calf and an active protease would destroy their protective properties. Kiermeier and Semper (15) have reported that there is a protease inhibitor present in cow's milk, presumably there to limit the action of the protease. Probably to learn most about the protease in milk a convenient means for separating protease and inhibitor should be found. However small the amount of protease, it can cause radical changes in milk or the casein prepared therefrom over a period of time. Warner and Polis (22) showed that the protease went with the casein when it was precipitated with acid. The protease was evident from the steady decrease that occurred in the viscosity of casein solutions. Based on the pH of maximum viscosity decreases, and increases in the solubility of the casein at pH 4.6, it was concluded that the optimum pH for the protease action was pH 8.5. The tests were performed in 2.0% borax solution, for sterility, with a 9.7% concentration of casein.

Kiermeier and Semper (15), on the other hand, found a pH optimum of 6.5. Their experiments were done with milk itself and the increase in solubility in trichloroacetic acid measured in experiments lasting 6 hr. Others (6) have not obtained a significant increase in solubility in this short period of time, although increases are obtained in longer periods of time. A more sensitive assay method for protease is badly needed for study of the protease in milk. The milk protease is relatively heat-sensitive. Kiermeier and Semper (15) reported that 158 F (70 C) for 2 min would inactivate it; Warner and Polis (22) found no protease in casein solutions heated at 176 F (80 C) for 10 min. Thus, pasteurization conditions would inactivate some of the protease and sterilization temperatures would certainly inactivate all of it. In view of the behavior of alkaline phosphatase with high-temperature, short-time pasteurization (HTST), the possibility of reactivation of the protease must be kept in mind. Reactivation after HTST sterilization temperatures is less likely, but it must be kept in mind in the production of HTST evaporated milk. It should be remembered that proteases can clot milk.

This quick look at four of the enzymes in milk shows that they contribute to its dynamic state, but apparently they may not pose a serious problem in the marketing of milk or milk products. There are, however, suspicious areas that require that further research be done on these enzymes to fully understand their relation to changes in the properties of milk.

SUMMARY

The action of the milk enzymes, xanthine oxidase, acid phosphatase, ribonuclease, and protease, on components of milk are reviewed. The possible influence of these enzymatic changes on the properties of milk is discussed.

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